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Ambrosia artemisiifolia control in agricultural areas: effect of grassland seeding and herbivory by the exotic leaf beetle *Ophraella communa*

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Abstract

Ambrosia artemisiifolia (common ragweed) is an invasive species native to North America and was accidentally introduced to Europe in the 19th century. Widespread in disturbed habitats, it is a major weed in spring-sown crops and it causes serious allergic rhinitis and asthma due to its allergenic pollen. The aim of this research was to analyse the effects of both competitive vegetation and herbivory by *Ophraella communa* to control *A. artemisiifolia* in an agricultural area of north-western Italy. Hayseed mixtures, both over-seeded over the resident plant community or after ploughing, when seeded before the winter season, were able to suppress the establishment of *A. artemisiifolia* as well as to reduce its growth in terms of plant height and inflorescence size. Defoliation of *A. artemisiifolia* by *O. communa* at the end of the growing season was conspicuous but most of the plants still produced flowers and seeds. However, significant *O. communa* attack was recorded for reproductive structures. As for non-target species, *O. communa* was mainly recorded on Asteraceae, with low density and low degree of damage. Reduction of inflorescence size due to competitive vegetation and damage to male flowers by *O. communa* may diminish the amount of available pollen. The results of this study may be useful for the implementation of management measures to control *A. artemisiifolia* in agricultural areas using mixtures of native species.

Keywords

Allergenic species, Invasive species, Competitive vegetation, Biological control, Agriculture, Common ragweed

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Introduction

The introduction of Invasive Alien Species (IAS) in a new region has multi-scale impacts on ecosystems and socio-economic implications for resident human communities (Branco et al. 2015, Early et al. 2016). For these reasons, multi-disciplinary approaches to study the consequences of IAS as well as implementing sustainable management options are required, with the final goal being to accomplish successful control measures and/or eradication (Harker and O'Donovan 2013). In Europe, *Ambrosia artemisiifolia* L. (common ragweed) is considered an extremely dangerous IAS, due to both its allergenic pollen that causes serious human diseases such as rhinitis and asthma (Ghiani et al. 2016) and its impact on crops yields that decrease when ragweed is abundant (Essl et al. 2015). The species, native to North America, was accidentally introduced into the wild in Europe around the middle of the 19th century (Gentili et al. 2017a) probably through contaminated seed stocks (birdseeds, corn, grain etc.; Brandes and Nitzsche 2006) and, from then, it spread exponentially in several countries (Chauvel et al. 2006, Ciappetta et al. 2016) and other continents (Montagnani et al. 2017). It is expected that the species will expand its range further due to its great dispersal ability and favoured by global warming (Cunze et al. 2013, Chapman et al. 2014, Leiblein-Wild et al. 2016, Skálová et al. 2017). Exchanges of contaminated crop seeds still represent an important vector for diffusion (Essl et al. 2015) but, despite the absence of specialised dispersal structures, *A. artemisiifolia* seeds are also spread by water (river flooding; Fumanal et al. 2007), animals and human activities (Chauvel et al. 2006, Vitalos and Karrer 2009, Von der Lippe et al. 2013, Montagnani et al. 2017). As an annual pioneer species, it colonises disturbed habitats, such as river corridors, roadside verges, ruderal and agricultural areas (Chauvel et al. 2006, Müller-Schärer et al. 2014, Gentili et al. 2015). To date, several control measures have been tested to promote its eradication, including chemical, physical and biological techniques. However, mowing and herbicides are still the most applied methods in agro-ecosystems (Buttenschøn et al. 2009, Milakovic et al. 2014).

Recently, in the framework of the EU-project SMARTER (EU COST action FA1203: *Sustainable management of* Ambrosia artemisiifolia *in Europe*, http://www.ragweed.eu), a multi-disciplinary team of researchers is performing several studies directed at creating innovative measures to control the species, according to different methods: mechanical, chemical, biological and ecological (Müller-Schärer et al. 2014, Bonini et al. 2017). With regard to ecological methods, "competitive vegetation" created from species-rich seed mixtures and vegetation succession are considered promising approaches to suppress the species' growth (Gentili et al. 2015, 2017b), while "biological control" through the insect *Ophraella communa* LeSage 1986 (Coleoptera: Chrysomelidae) is currently under evaluation (Müller-Schärer et al. 2014, Lommen et al. 2017a, 2017b, Bonini et al. 2017, Sun et al. 2017). The insect, whose presence was recently reported for Europe (Müller-Schärer et al. 2014), is used as a successful biological control agent in China, together with *Epiblema strenuana* (Zhou et al. 2014).

As for competitive vegetation, *A. artemisiifolia* is well known for being able to rapidly occupy empty niches across its invasion range; particularly, being an aggres-

sive early coloniser of open disturbed habitats (Gentili et al. 2015) and abandoned crop fields (Maryushkina 1991), it takes advantage of the "priority effect" (Dickson et al. 2012) which allows it to better outcompete other species and, thus, inhibits their establishment and growth (Young et al. 2001, Ortmans 2016). In turn, enforcing competitive vegetation, seeding both native (hayseed) and commercial seed stocks, has been demonstrated to inhibit ragweed germination and growth, to increase biodiversity (hayseed) and to be effective in recovering ruderal habitats (Gentili et al. 2015). Such a method has yet to be tested in agricultural and/or protected areas, contexts where methodological issues, such as how to schedule periods of seeding, are important in order to understand the way of limiting its priority effect advantages and consequently maximising the controlling effect on *A. artemisiifolia*. Inevitably the seeding strategy (i.e. restoration) is habitat-dependent due to local environmental and biotic filters and needs to be calibrated on recipient environmental types as its effectiveness is strictly connected to local ecosystem conditions (Funk et al. 2008).

With regard to biological control, *O. communa* is a multi-voltine leaf beetle originally from North America (Futuyma and McCafferty 1990). It was first recorded in Europe in 2013, when the species was found in northern Italy (Lombardy, Piedmont and Emilia-Romagna regions) and in southern Switzerland (Ticino Canton; Bosio et al. 2014, Müller-Schärer et al. 2014). Due to its high dispersal ability (potentially up to 329 km/year; Yamamura et al. 2007), it was expected to rapidly expand its European range and, by 2013, was already covering an area of 20000 km^2 (Müller-Schärer et al. 2014, Lommen et al. 2017b). *O. communa* larvae and adults preferentially feed on *A. artemisiifolia* and they can completely defoliate the plant to death prior to seed production when the initial density is high enough (Guo et al. 2011, Zhou et al. 2014). Also in Italy and Switzerland, *O. communa* was observed to reach densities high enough to kill *A. artemisiifolia* plants before flowering (Müller-Schärer et al. 2014) and beetles were seen to cause damage to male flowers, with negative effects on pollen production (Bonini et al. 2016). The recent spread of the insect is considered to be the potential explanation for the low levels of *A. artemisiifolia* pollen in the Milano area recorded during 2013 and 2014 (Bonini et al. 2015, 2016).

One of the greatest concerns when choosing a biocontroller is clearly connected to the risk for non- target species to be attacked by the agent (Louda et al. 2003). As for *O. communa*, the insect was reported on plant species different from *A. artemisiifolia*, such as other ragweed *taxa* and relatives mainly belonging to the tribe of Heliantheae (Tamura et al. 2004, Watanabe and Hirai 2004, Yamanaka et al. 2007, Cao et al. 2011, Müller-Schärer et al. 2014). Risk assessments of *O. communa*'s attack on the cultivated sunflower *H. annuus* gave controversial results. Palmer and Goeden (1991) rejected the beetle as a biocontroller for Australia because, in laboratory tests, it can complete its life cycle on sunflower while, recently, the possibility of an *Ophraella*'s attack on *H. annuus* in the field was considered negligible (Dernovici et al. 2006, Cao et al. 2011, Zhou et al. 2011). Most of the studies on the potential ability of *O. communa* to choose new host plants in introduced areas were conducted on weeds or species of commercial interest (Palmer and Goeden 1991, Watanabe and Hirai 2004, Dernovici et al. 2006,

Cao et al. 2011, Zhou et al. 2011, Lommen et al. 2017b). The risk to native flora was rarely taken into account.

Taking advantage of the recent accidental introduction of *O. communa* in Italy, the present work analyses the effects and *modus operandi* of both competitive vegetation and herbivory by *O. communa*, as well as their possible additive or divergent effect, on the management and control of *A. artemisiifolia* in an agricultural protected area. Specifically, the aims of this study were to:

a) assess the effectiveness of seeding competitive native vegetation to control *A. artemisiifolia*; in particular, to assess the priority effect advantages by testing two different seeding periods;
b) evaluate the damage to *A. artemisiifolia* caused by natural population of *O. communa* in an area of the European range where both the plant and the insect are present at very high densities;
c) detect the presence of *O. communa* on resident non-targets species (i.e. species other than *A. artemisiifolia*) and its damage to these plants, in order to assess if potential future use of the beetle as a biological agent could contrast with seeding competitive vegetation.

Methods

Study area and experimental design

The study was carried out in the "Alto Milanese" Park (359 ha), a protected area of local interest sited in northern Italy, approximately 28 km north-western from the city of Milan (45°35'38.20"N, 8°51'52.61"E). The park is located in one of the most invaded areas by *A. artemisiifolia* (Gentili et al. 2015) and its surface is mainly covered by cropped fields (60.2 %; Parco Alto Milanese 2007). Woodlands (17 %), mostly dominated by *Prunus serotina* Ehrh. and *Robinia pseudoacacia* L., fallow fields (1.6 %) and hedgerows (3.8 %) are also present.

In 2014, three sites with comparable soil properties and a seed bank of *A. artemisiifolia* (Suppl. material 1: Table S1, Figure S1), were selected inside the park: (1) a short-rotation clover field (X: 45°35'42.37"N, 8°51'52.99"E), (2) an oat field (Y: 45°35'54"N, 8°52'9"E) and (3) a short-rotation meadow (Z: 45°35'37"N, 8°52'14"E). Each site contained three squared plots of 100 m^2, separated by 1 m buffer, for a total number of 9 plots. In each site, the following treatments were set up:

(a) *Control - not seeded* (C): the plot was harrowed and ploughed no deeper than 15 cm and then left to spontaneous vegetation colonisation, without sowing any herb layer;
(b) *Hayseed* (Hs): the plot was harrowed and ploughed no deeper than 15 cm and then seeded with hayseed at a density of about 20 g/m^2. In June 2013, a mowed mesophilous grassland dominated by *Arrenatherum elatius* (L.) P. Beauv. ex J. & C. Presl close to the study area was selected as a donor grassland for hayseed collection. The

most frequent species of the mixture besides *A. elatius* were: *Achillea millefolium* L., *Centaurea nigrescens* Willd., T*rifolium pratense* L. and *T. repens* L. Once dried, hayseed was prepared in accordance with the protocols of the Native Flora Centre of the Lombardy Region (Ceriani et al. 2011);

(c) *Over-seeding hayseed* (Ov): the plot was only superficially harrowed and over-seeded with hayseed at a density of about 20 g/m^2.

In 2014, these treatments were applied in March (late seeding), then the experiment was repeated in the same sites during 2015, the only difference being that soil was prepared and hayseed sown in October 2014 (early seeding).

The proposed experimental approach is quite different from that published in Gentili et al. (2017b) especially: a) the current work was done in protected arable areas, suffering from the expansion of *A. artemisiifolia*; b) different seeding periods were applied in 2014 and 2015; c) different techniques for soil treatment were used.

Data collection

Vegetation

In 2014 and 2015, vegetation data were collected in three 2 m × 2 m quadrats randomly chosen within each plot, at least 1 m from the edge. The following parameters were measured in June for the vegetation cover other than *A. artemisiifolia* and in September for the weed abundance and traits (on 30 randomly selected plants, when present) (Gentili et al. 2015): (a) vegetation cover: percentage vegetation cover other than *A. artemisiifolia*, visually estimated; (b) species abundance: number of individuals of *A. artemisiifolia*; (c) vegetative traits: plant height (cm), measured from the plant collar to the apex; plant width (cm), measured as the maximum width of an individual; maximum leaf length (cm), measured from the petiole to the leaf apex; (d) reproductive traits (i.e. pollen production proxies): maximum size of male composite inflorescence, i.e. spike (mm); total number of male inflorescences.

In order to assess the effect of hayseed cover on soil temperature at the beginning of the vegetative period, two dataloggers (model TransitempII, Magditech) were placed in control and hayseed treatments at site Z during April 2015, corresponding to the germination of *A. artemisiifolia*. Dataloggers were buried at a depth of 10 cm and the temperature was measured daily, with an interval of 30 minutes.

O. communa presence and damage to A. artemisiifolia

In mid-September 2015, when damage caused by *O. communa* to *A. artemisiifolia* is usually at its maximum (Miyatake and Ohno 2010, Fukano et al. 2013) and the weed is at the end of its growing season (MacKay and Kotanen 2008, MacDonald and Kotanen 2010a), the following data were recorded for 25 plants (when present) in each

plot: (a) if individuals were mature, i.e had raceme longer than 1 cm or had female structures or seeds formed (http://www.ragweed.eu); (b) the number of individuals of *O. communa* in every life stage (i.e. egg batches, larvae, pupae and adults); (c) the damage, visually assessed and expressed as a percentage of missing tissue, caused by *O. communa*, separately for leaves, stems, reproductive structures and for the whole plant.

O. communa presence and damage to non-target species

In each site, during the summer of 2015, *O. communa* presence on non-target plants was monitored in an area of about 600 m^2 that included the plots and the surrounding vegetation. Non-target species were selected on the basis of the hayseed composition (i.e. most frequent species) and of floristic surveys of common plants in the area and included genus and species belonging to six different families: (1) Asteraceae: *Achillea millefolium, Artemisia verlotiorum* Lamotte, *Centaurea* sp. pl. (*C. montana* L. and *C. nigrescens* Willd.), *Erigeron annuus* (L.) Pers.; (2) Poaceae: *Arrhenatherum elatius, Holcus lanatus* L., *Lolium* sp. pl. (*L. multiflorum* Lam. and *L. perenne* L.), *Sorghum halepense* (L.) Pers.; (3) Polygonaceae: *Persicaria maculosa* Gray, *Polygonum* sp. pl. (*P. arenastrum* Boreau, *P. lapathifolium* L.); (4) Chenopodiaceae: *Chenopodium album* L.; (5) Fabaceae: *Trifolium* sp. pl. (*T. pretense, T. repens*); and (6) Papaveraceae: *Papaver rhoeas* L.

From early June to the end of September 2015, the following data were recorded fortnightly for 5 individuals of each non-target species (when present) in every sites: (a) phenological stage, i.e. vegetative, flowering or seedling; (b) presence/absence of *O. communa* and the number of individuals in every life stage (i.e. egg batches, larvae, pupae and adults); (c) when *O. communa* was present, the damage, visually assessed and expressed as a percentage of missing tissue, potentially caused by the beetle, separately for leaves, stems, reproductive structures and for the whole plant. Damage was evaluated only when the insect was seen on the plant to minimise the possibility of mistakenly assigning to *O. communa* a feeding event due to other herbivores. Moreover, in order to increase the probability of *O. communa* encounter on non-target species, in each session, plants were randomly chosen for observation so that the same individuals were rarely sampled.

In order to have comparison data of the beetle presence and attack on its primary host throughout the season, 10 *A. artemisiifolia* plants were contemporarily monitored in each site, with the same method described above for the non-target species. In every session, the beetle density (i.e. number of egg batches, larvae, pupae and adults) was also estimated in 11 quadrats of 1 m^2 homogeneously distributed inside the 600 m^2 area.

Data analysis

Vegetation

Prior to any statistical analysis, vegetation data, collected in the three quadrats, were pooled for each plot. Differences in vegetation cover of species other than *A. artemisiifolia*

and in vegetative and reproductive traits of *A. artemisiifolia* plants in different treatments and years were tested by Linear Mixed Effects models (LME). Treatment and year were fitted as interacting fixed factors, while the site was fitted as a random effect. When necessary, the data were log-transformed to normalisation. The difference in number of *A. artemisiifolia* individuals between treatments and years was tested using a negative binomial Generalised Linear Mixed Models (GLMM) model (to correct for over-dispersion) with the same structure of LMEs described above.

Soil temperature was compared between control and hayseed treatments by means of a *t* test.

O. communa on A. artemisiifolia and non-target species

Differences in damage to leaf and reproductive structures of *A. artemisiifolia* caused by *O. communa* and in the number of adult beetles per plants between treatments were tested by GLMMs, with the treatment as fixed factor and the site as random effect. Data on damage was arcsin-transformed [$Y = \text{asin}(\sqrt{(0.01*y)})$]) and modelled with a Gaussian distribution, while data on density was modelled with a negative binomial distribution to correct for over-dispersion. The difference in *A. artemisiifolia* height related to leaf damage caused by *O. communa* was tested only in control plots, where the weed growth was not influenced by hayseed competition and only scarcely influenced by spontaneous vegetation: a LME model was constructed, with leaf damage as continuous fixed factor and the site as categorical random effect.

Data on *O. communa* presence and damage on non-target species were analysed only qualitatively and cumulated throughout the season, due to low number of records of the beetle on species other than *A. artemisiifolia*. Moreover, data were cumulated over the three sites, as *O. communa* density was similar during the study period (mean ind/plant: site X = 14.4, site Y = 13.6, site Z = 15.8; $X^2 = 0.5$, DF = 2, NS).

All statistical analyses were performed using R version 3.3.2 (R Core Team 2016) and *post-hoc* tests by means of the packages "lsmeans" (Lenth 2016) and "multcomp" (Hothorn et al. 2008).

Results

Vegetation cover and *A. artemisiifolia* abundance

Vegetation cover of species other than *A. artemisiifolia* did not exhibit differences amongst treatments within the two years of observation ($F_{2,10} = 1.84$, p = NS). On the contrary, vegetation cover significantly varied between 2014 and 2015 in all treatments (Figure 1a): in C from 49.3 % to 87.3 % ($t = -3.9$, $p < 0.003$); in Ov from 68 % to 92.7 % ($t = -2.53$, $p < 0.03$); in Hs form 57.7 % to 91.3 % ($t = -3.46$, $p < 0.006$).

In 2014, following the "late seeding", the number of individuals of *A. artemisiifolia* did not show any differences amongst treatments (Figure 1b). On the contrary, in

2015 (early seeding), C exhibited a higher number of individuals than both Ov and Hs ($z_{C\ vs\ Ov} = 5.41$, $p < 0.001$; $z_{C\ vs\ Hs} = 5.23$, $p < 0.001$). Comparing the two observation years, the number of *A. artemisiifolia* individuals greatly decreased in Ov ($z = 4.76$; $p < 0.001$) and Hs ($z = 4.53$, $p < 0.001$).

Daily soil temperature was significantly different during April 2015 in C and Hs treatments (16.6 ± 3.1 °C and 14.8 ± 3.1 °C, respectively; $t = 15.2$; $p < 0.001$).

A. artemisiifolia traits

With regard to plant height, in 2014, the species showed quite a large size, reaching more than 1 m for the mean in all treatments that differed significantly from each other (Figure 1c): the highest individuals were found in Hs while the shortest ones were in Ov ($t_{C\ vs\ Ov} = 3.98$, $p < 0.001$; $t_{C\ vs\ Hs} = -3.31$, $p < 0.003$; $t_{Ov\ vs\ Hs} = -7.29$, $p < 0.001$). In 2015, C exhibited a significantly higher size than both Ov ($t = -12.85$, $p < 0.001$) and Hs ($t = 15.9$, $p < 0.001$). Comparing the treatment trends in the years 2014 and 2015, they exhibited a strong reduction in plant height in the second year (C: $t_{2014\ vs\ 2015} = 18$, $p < 0.001$; Ov: $t_{2014\ vs\ 2015} = 11.94$, $p < 0.001$; Hs: $t_{2014\ vs\ 2015} = 20.037$, $p < 0.001$). This reduction was particularly marked in Hs where the plant height diminished to 94.1 ± 4.7 cm.

With regard to inflorescence size, in 2014, it was different between treatments Ov and C ($t = 4.25$, $p < 0.001$; Figure 1d) and between Ov and Hs ($t = 4.91$, $p < 0.001$). In 2015, the C plots showed larger inflorescences than those of Ov and Hs ($t_{C\ vs\ Ov} = 4.53$, $p < 0.001$; $t_{C\ vs\ Hs} = 6.41$, $p < 0.001$). In addition, in this case, comparing the two observation years, the inflorescence size significantly decreased in all treatments (C: $t_{2014\ vs\ 2015} = 9.86$, $p < 0.001$; Ov: $t_{2014\ vs\ 2015} = 4.32$, $p < 0.001$; Hs: $t_{2014\ vs\ 2015} = 8.78$, $p < 0.001$).

Regarding the other collected plant traits (plant width, maximum leaf length and number of male inflorescences), similar trends to those of inflorescence size were observed. In particular, in 2015, C differed from Ov and Hs, while comparing the same traits in 2014 and 2015, reductions in size and number was recorded (see Suppl. material 1: Figure S2).

O. communa presence and damage to *A. artemisiifolia*

Overall, 192 plants of *A. artemisiifolia* were observed: 75, 75 and 42 individuals in C, Ov and Hs treatments, respectively. The lower number of plants monitored in Hs was due to the low density of the weed in those plots (see paragraph "*Vegetation cover and A. artemisiifolia abundance*"). Of the sampled individuals of *A. artemisiifolia* in mid-September, 94.8% were mature, i.e. with reproductive structures formed.

In total, 3267 *O. communa* were found on *A. artemisiifolia* plants; most of all were adults (76.3 % vs 17.8 % larvae, 3 % pupae, 2.9 % egg batches). All plants except one (in Ov treatment) were attacked by the insect, reporting damage on about 72 % of the

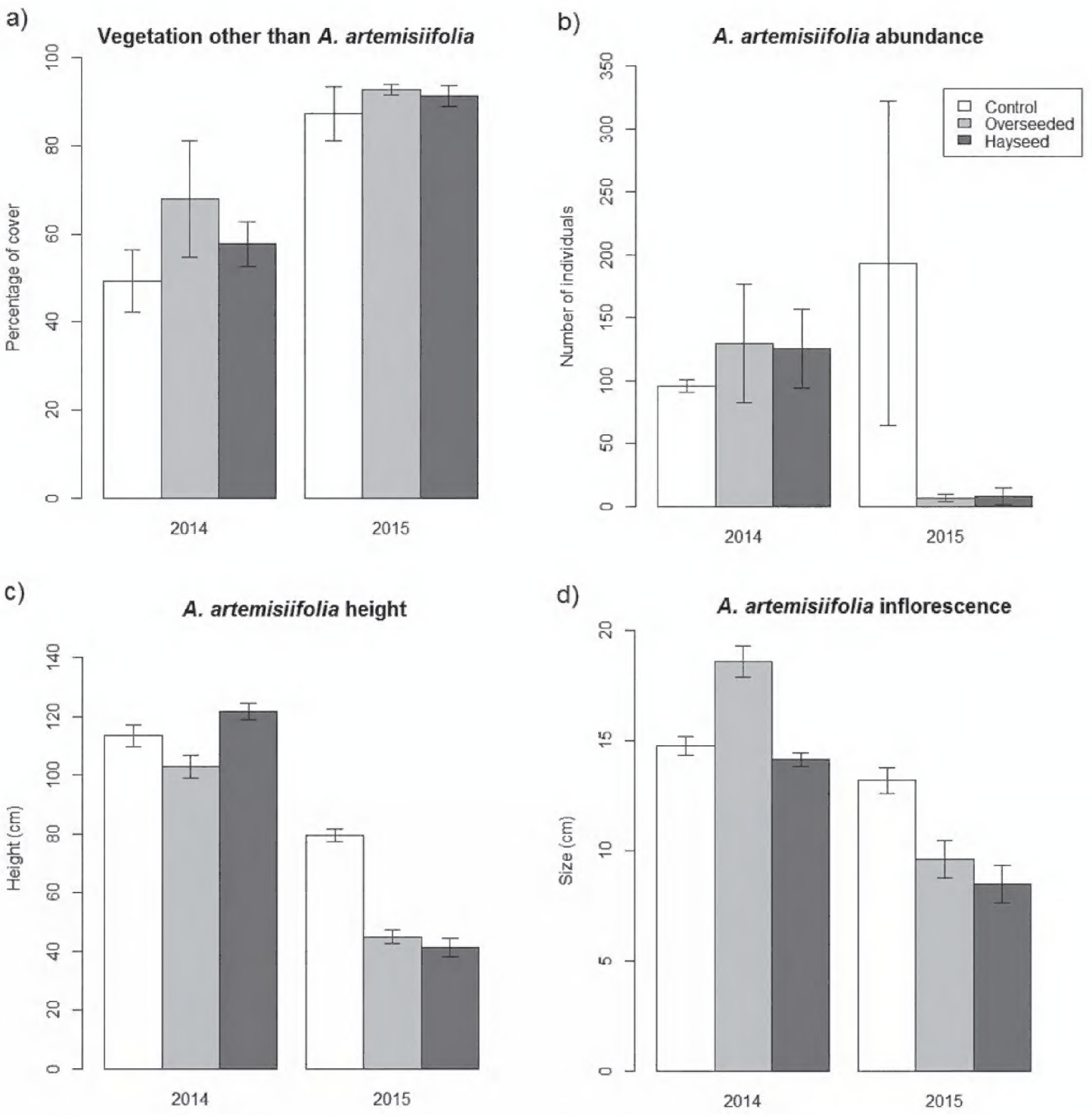

Figure 1. *A. artemisiifolia* abundance and traits in experimental plots. Mean values (± SE) for **a** percentage cover of vegetation other than *A. artemisiifolia* **b** number of individuals **c** plant height and **d** inflorescence size of *A. artemisiifolia* in the three treatments (control, over-seeded and hayseed) in September 2014 and 2015.

whole tissues (min.: 2 %, max.: 99 %). Of the attacked plants, all had conspicuous damage on leaves and 90 % on reproductive structures (i.e. male inflorescences and seeds; Table 1). Damage on the stem was reported for 30.7 % of the plants but was not influential as it concerned, on average, only 4 % of the tissues.

The number of adults per plants was significantly lower in Hs plots with respect to C and Ov plots and also in Ov plots with respect to those in C ($z_{\text{Hs vs C}} = -11.54$, $p < 0.001$; $z_{\text{Hs vs Ov}} = -7.83$, $p < 0.001$; $z_{\text{Ov vs C}} = -5.37$, $p < 0.001$; Table 1). However, damage on *A. artemisiifolia* leaves in the three treatments was similar, while damage on reproductive structures in C and Hs plots was higher than the damage in Ov ($z_{\text{C vs Ov}} = 3.96$, $p < 0.001$;

Table 1. *O. communa* density and damage on *A. artemisiifolia*. Mean values (± SE) for adult *O. communa* per plant and *A. artemisiifolia* damage on leaves and reproductive structures in the three treatments (C: control, Ov: over-seeded and Hs: hayseed) in September 2015, with X^2 / F tests results.

	C	Ov	Hs	X^2 / F	DF	p
Adult/plant	25.2 (3.4)	7.7 (1.8)	0.6 (0.2)	135.72	2	<0.001
Leaf damage (%)	74.6 (2.9)	69.6 (3.6)	74.3 (4.1)	2.87	2,187	n.s.
Repr. structure damage (%)	53.9 (3.9)	37.2 (3.7)	53.4 (5.3)	12.59	2,187	<0.001

$z_{\text{Hs vs Ov}} = 4.43$, $p < 0.001$; Table 1). In control plots, where *O. communa* was the only manifest controlling factor for the weed, the height of *A. artemisiifolia* was negatively related to leaf damage (coeff. = -0.54 ± 0.08, $F_{1,71} = 41.41$, $p < 0.001$).

O. communa presence and damage to non-target species

In total, 1255 non-target and 269 *A. artemisiifolia* plants were monitored during the summer of 2015. Non-target individuals (461, 395 and 399) were in vegetative, flowering and seeding stages, respectively.

O. communa was recorded on 107 (8.5 %) non-target and 181 (67.3 %) *A. artemisiifolia* plants, with a total number of 215 and 1050 individuals, covering all life stages, respectively. The number of *O. communa* per plant on non-target species, averaged throughout the season, is reported in Figure 2 (min. on *Holcus lanatus*: 0.01 ind/plant; max. on *Artemisia verlotiorum*: 0.54 ind/plant; reference value on *A. artemisiifolia*: 3.9 ind/plant). The number of *O. communa* per plant in each sampling session is reported in Suppl. material 1: Table S2; no increasing trend in insect number on non-target species was observed throughout the summer. On 4 non-target species (*Lolium* sp. pl., *Papaver rhoeas, Persicaria maculosa, Polygonum* sp. pl.), no *O. communa* was detected.

Of the total observation of *O. communa* on non-target plants, most were adults (87.4 %), while only 2.8 % were egg batches, 3.7 % larvae and 6.1 % pupae. Oviposition were recorded on *Artemisia verlotiorum* (n = 1), *Centaurea* sp. pl. (n = 2) and *Trifolium* sp. pl. (n = 3). Only on *Artemisia verlotiorum* all the stages were recorded (egg batches: 1; larvae: 5; pupae: 11, adults: 86).

On 6 of the 9 species where *O. communa* was present, damage was observed (Table 2). For each species, less than 7 % of monitored individuals were attacked throughout the season and only on *Centaurea* sp. pl. the mean damage was higher than 10 % (Table 2). Damage on non-target species was recorded only on leaves.

Discussion

Vegetation

This study ascertained the effectiveness of seeding competitive vegetation from native species mixture of hayseed, both over-seeded over the resident plant community or

Table 2. *O. communa* density and damage on non-target species. Non-target species with total number of monitored individuals, percentage of individuals with *O. communa* and with damage and mean percentage damage on observed and attacked plants during summer 2015.

Species	No. plants	% of plants with *O. Communa*	% of plants with damage	Mean damage on all observed plants (%)	Mean damage on plants with damage (%)
Achillea millefolium	151	7.3	4.0	0.2	4.2
Artemisia verlotiorum	189	22.8	6.3	0.2	3.9
Centaurea sp.pl.	220	8.6	6.8	1.2	17.1
Chenopodium album	100	14	2.0	0.1	2.5
Erigeron annuus	68	5.9	5.9	0.2	3.0
Trifolium sp.pl.	195	5.6	4.6	0.3	6.0
Reference on *Ambrosia artemisiifolia*	269	67.3	82.9	24.8	29.9

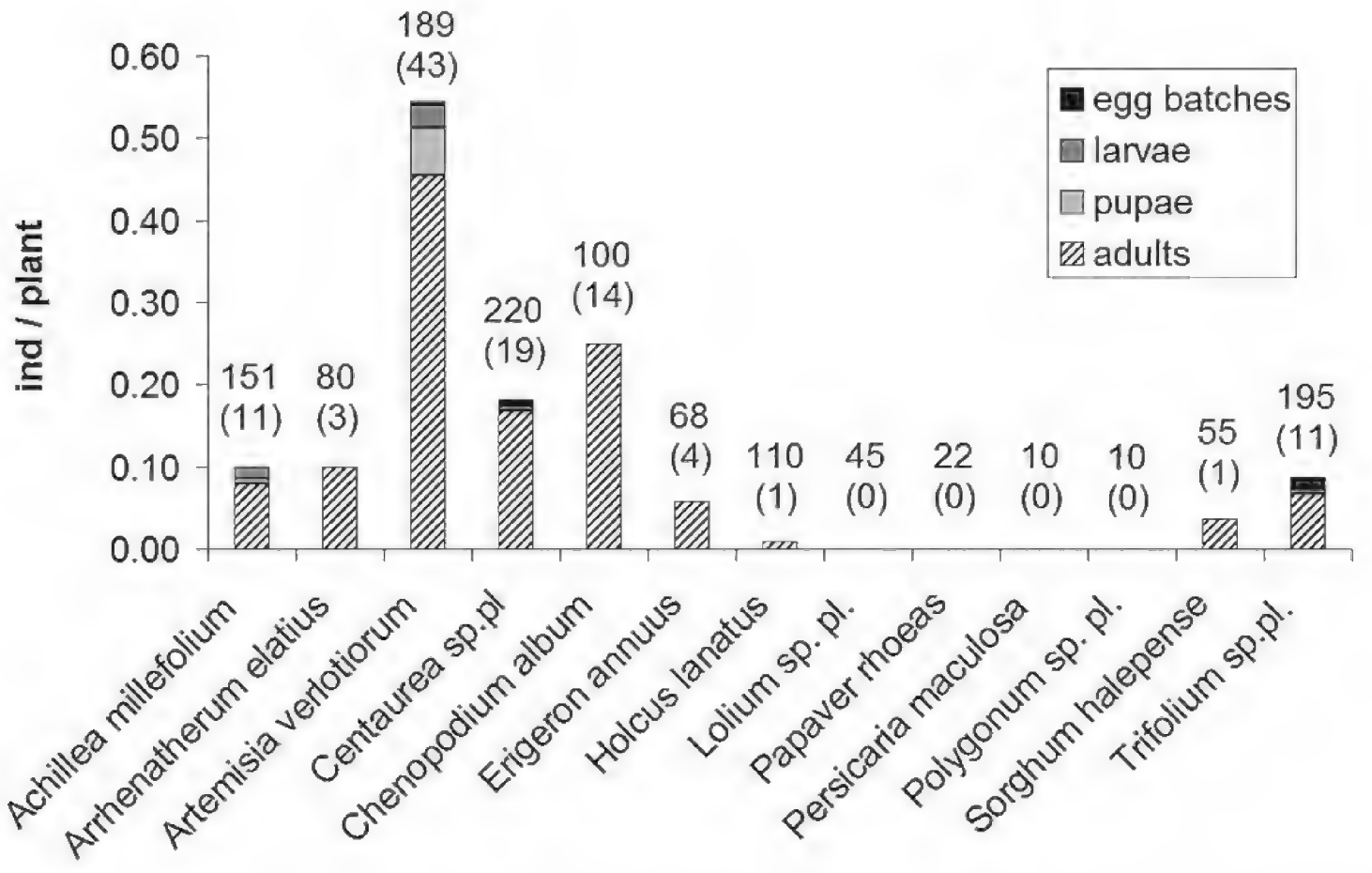

Figure 2. *O. communa* density on non-target species. Number of *O. communa* per plant on non-target species monitored during summer 2015. On the top of each bar is reported the number of observed plants and, in brackets, the plants with *O. communa* presence. Reference value of *O. communa* presence on *A. artemisiifolia* plants (n = 269, 181 with *O. communa*) are: 1.01 egg batches/plants: 0.69 larvae/plants, 0.21 pupae/plants, 1.99 adults/plants).

after ploughing, in controlling *A. artemisiifolia* in an agricultural area. Particularly, it confirmed the trend observed in a ruderal quarry habitat (Gentili et al. 2015, 2017b), that competitive vegetation was able to suppress the establishment and growth of *A. artemisiifolia* as well as possibly contributing to reduce the soil seed bank of the species and its pollen production (i.e. lower number of plants and reduced inflorescences). The competitive ability of *A. artemisiifolia* in continuously disturbed sites such as agricultural

areas has been recognised as being a key factor in promoting its invasion success (Kazinczi et al. 2008, Bullock et al. 2012). In these results, a high vegetation cover in seeded areas, holding an increased number of competitor species filling vacant niches, controlled or suppressed the species. This could be due, from one side, by limiting available resources (light and nutrients) and on the other, by modifying local environmental conditions (Katz et al. 2014). For instance, soil temperature was lower in highly vegetated treatments (i.e. hayseed): lower temperature may delay germination of *A. artemisiifolia* and enable the competitors to grow more and before the weed. In fact, the recent study of Skálová et al. (2015) highlighted that temperature is a main determinant of *A. artemisiifolia* distribution, especially influencing the species' germination (Leiblein-Wild et al. 2014). For all such reasons, the performance of *A. artemisiifolia* was poorer where vegetation cover was higher. Even if the percentage cover of other species was also relatively high in control plots in 2015, they showed a very limited growth in height under the canopy of *A. artemisiifolia.*

In addition, testing in the field two different seeding periods (i.e. an early and a late seeding period), allowed the verification of different priority effect advantages for this invasive species. Seeding the hayseed after the winter season (late seeding) allows the earlier development of *A. artemisiifolia*, as it gives it a competitive advantage with a temporal priority effect. This advantage may lead to a different community structure dominated by *A. artemisiifolia*. Indeed, when it starts to grow simultaneously, the weed is able to growth rapidly and out-compete native species. This kind of performance was also observed by Ortmans (2016) in a greenhouse experiment. On the other hand, anticipating the seeding of hayseed before the winter season (early seeding), native species start to grow before the weed, eliminating or strongly reducing the temporal priority effect for *A. artemisiifolia* and shifting to the native species assemblage (i.e. hayseed).

Priority effects of alien species have been previously investigated in several plant communities in the context of habitat restoration and control of alien species, with the final aim being to encourage the competitive effect of native species over invasive ones (Vaughn and Young 2015). Studying the role of priority in shaping community composition can address management activities and the choice of native plant assemblages able to inhibit invasive plant species (Zefferman 2015).

O. communa on *A. artemisiifolia*

A high number of *O. communa* heavily feeding on both leaves and reproductive structures of *A. artemisiifolia* was observed in the study area, as already reported for other sites in northern Italy (Bosio et al. 2014, Müller-Schärer et al. 2014, Bonini et al. 2016). Damage on leaves involved, on average, 73 % of the tissues and caused a significant reduction in plant size, but about 95 % of *A. artemisiifolia* still had flowers and/or seeds. This was not unexpected as defoliation by herbivores is found to reduce plant height and the number of branches (Guo et al. 2011) but not to affect the ability

of the weed to fructify (MacDonald and Kotanen 2010b). However, 90 % of sampled plants reported damage on around 40–50 % of the reproductive structures tissue (i.e. male inflorescences and seeds). Damage to racemes is of great importance in terms of biocontrol because it may reduce the amount of available pollen thus benefiting the allergic human population (Bonini et al. 2015, 2016).

With regard to the treatments, *O. communa* density was very variable, ranging from an average of 25.2 adults per plants in control plots to 0.6 adults per plants in hayseed plots. Despite this difference, damage to leaves was similar in C, Ov and Hs; damage to reproductive structures was also comparable and quite high, even if it was lower in Ov than in the other two treatments. This likely indicates an ability of the insect to move between *A. artemisiifolia* patches and find its primary host even when the plant is at very low density. *O. communa* is considered to have high dispersal ability (Yamamura et al. 2007). Tanaka and Yamanaka (2009) estimated that it can potentially fly a distance of 25.4 km in 23 hours. When the beetle finds a new *A. artemisiifolia* plant, it is able to severely damage it; if the attack is massive, the beetle and its next generations are obliged to move again to search for other plants, both for feeding and reproduction (Yamazaki et al. 2000, Tanaka and Yamanaka 2009). These results highlighted that, during summer 2015, the *O. communa* density was sufficiently high to force the species to move also to Ov and Hs plots, where *A. artemisiifolia* presence was low; there, the damage caused, combined with low availability of other primary hosts in the immediate vicinity, likely led the beetle to leave these patches, justifying the low number of insects per plants observed in these plots at the end of the season.

Despite the apparent overall high number of beetles and the conspicuous damage caused to leaves and reproductive structures, in the study area *O. communa* did not naturally reach the minimum density crucial for the suppression of *A. artemisiifolia* population in the short term and parts of the plant which were able to produce seeds survived, even if climate during summer 2015 was favourable for the beetle development. The mean temperature during daylight was between 25–30 °C (June: 24.7 °C, July: 29.8 °C, August: 25.2 °C; U.O. Meteoclimatologia 2017), which is suggested as an optimum range for *O. communa* population growth (Zhou et al. 2010). In fact, studies conducted in China, where *O. communa* is used as a successful biocontroller of *A. artemisiifolia*, demonstrated that the beetle effectiveness is highly density-dependent (Guo et al. 2011, Zhou et al. 2014). Moreover, the number of beetles should be higher with increasing plant height. For example, Guo et al. (2011) reported that ≥ 1.07 and ≥ 12 adults per plant, at early and late growth stage, respectively, should be released to cause the complete defoliation of the weed and its death prior to fructification. Gard et al. (2013) and Zhou et al. (2014) also suggested that biocontrol of *A. artemisiifolia* should include various specialised enemies, whose joint combination that weaken different parts of the plant (e.g. roots, leaves, flowers) could prevent the reallocation of its resources on undamaged structures. Despite determining the ability of *O. communa* to cause damage to male flowers with potential reduction in pollen release, more investigations are certainly needed to understand if, in Italy, the natural density of the beetles, even if not able to suppress *A. artemisiifolia* population in the short term, will be able

to decrease seed production and, consequently, reduce the weed abundance in the medium-long term or if inundative releases, maybe in combination with other agents, are necessary in the case the insect is elected as a suitable biocontroller.

O. communa on non-target species

The risk for non-target species was potentially high in the area which was monitored. The number of *O. communa* was conspicuous and, at the end of the season, *A. artemisiifolia* defoliation was relevant; therefore, there were suitable conditions for movement of the beetle to other hosts. However, *O. communa* was recorded only on 8.5% of observed non-target plants. Beetle detection started from the first sampling session, in early June, but no trend was observed throughout the summer, neither was there an increase in September, when food shortage caused by *A. artemisiifolia* exploitation could have forced migration to other species.

Greater incidence of *O. communa* was recorded on species belonging to the family of Asteraceae containing relatives of *A. artemisiifolia* (*Achillea millefolium*, *Artemisia verlotiorum*, *Centaurea* sp. pl., *Erigeron annuus*), but also on *Chenopodium album* (Chenopodiaceae) and *Trifolium* sp. pl. (Fabaceae). Similar results were obtained in other studies where the insect, when reported on plant species different from *A. artemisiifolia*, was present on relatives of the weed (e.g. *A. trifida* L. and *A. psilostachya* DC., *A. cumanensis* Kunth, *Xanthium* sp. pl., *Heliantus* sp pl., *Iva* sp. pl. and *Parthenium* sp. pl.; Palmer and Goeden 1991, Tamura et al. 2004, Watanabe and Hirai 2004, Yamanaka et al. 2007, Cao et al. 2011). On the other 7 plant species sampled, no beetle was detected (*Lolium* sp. pl., *Papaver rhoeas*, *Persicaria maculosa*, *Polygonum* sp. pl.) or only adults were present with no trace of feeding (*Arrhenatherum elatius*, *Holcus lanatus*, *Sorghum halepense*), suggesting casual wandering of the insect (Yamazaki et al. 2000). When damage was present, it was on a very low percentage of individuals (never higher than 7%), on average, on no more than 6% of the tissues and always located on leaves, even if around 50% of the plants had flowers or seeds. Only on *Centaurea* sp. pl., the mean damage was higher, due to a few events where attack resulted on around 50 % of the leaf tissues. Particularly interesting is the observation of quite a high number of plants with *O. communa* presence (23 %) in all life stages on *Artemisia verlotiorum*, another exotic species close to *A. artemisiifolia*. Elsewhere in Italy, adult beetles were found on other Asteraceae (*X. strumarium*, *H. tuberosus*, *Erigeron canadensis* L. and *Dittricha graveolens* (L.) Greuter), some of which were feeding but not causing significant damage to the plants (Bosio et al. 2014, Müller-Schärer et al. 2014).

A limitation for this study is that *O. communa* was not directly observed feeding on *A. artemisiifolia* and this could lead to false positives (Palmer and Goeden 1991). However, it was attempted to contain mistakes by assessing damage only on plants where *O. communa* was present and where the feeding trace was similar to those left by the beetle. Consequently damage was recorded mainly on Asteraceae, that is a likely result

as relatives of *A. artemisiifolia* have already been reported as alternative sub-optimal hosts of *O. communa* (Yamazaki et al. 2000, Cao et al. 2011). Moreover, when damage was recorded, *O. communa* was often found at life stages other than adult, suggesting some kind of use by the insect and not only casual movements.

In the end, the overall risk for the non-target species monitored in this study seems small; the density of *O. communa* and damage on plants resulted as low and can be considered as occasional events. On the contrary, *O. communa* showed a strong preference for its primary host, *A. artemisiifolia*; beetle number, percentage of attacked plants and feeding were higher for *A. artemisiifolia* compared to non-target species. It has already been demonstrated that when *A. artemisiifolia* is in sufficient number to sustain *O. communa* population, the beetle prefers to complete its life cycle on its primary host (Yamazaki et al. 2000, Cao et al. 2011). Moreover, Dernovici et al. (2006) has seen that, even if all stages of *O. communa* can survive on species other than *A. artemisiifolia,* such as sunflowers, the population collapses within a few generations. However, it cannot be ignored that egg batches, larvae and pupae were also present on non-target plants, suggesting that specific laboratory and field tests on oviposition preference and larval development on non-target species should be conducted to precisely evaluate the risk of an *O. communa* shift in the case of absence of *A. artemisiifolia*.

Management implications

This work is one of the first that investigated, with an interdisciplinary approach, the effects of both competitive vegetation and herbivory by *O. communa* in contrasting the alien invasive species *A. artemisiifolia* in a protected agricultural, highly invaded, area.

Regarding competitive vegetation, during the implementation of hayseed (or seed mixtures), the key factors for controlling/suppressing the weed will be: (1) the seeding period before the winter season and (2) a gap-free vegetation cover. After adopting competitive vegetation, *A. artemisiifolia* decreases in abundance and reproductive potential (i.e. inflorescence size) and consequently, its allergenic impact could also be strongly reduced. Further studies will be needed to clarify the long-term effect on seed production and soil seed bank. This method is particularly suitable for agricultural protected areas where the use of herbicide is not allowed or discouraged.

With regard to herbivory, the crushing impact of *O. communa* on *A. artemisiifolia* is confirmed: severe damage to reproductive structures (racemes) was observed, probably conditioning the amount of released pollen and the allergenic potential of *A. artemisiifolia* populations (Bonini et al. 2015, 2016). However, *O. communa* was not able to kill *A. artemisiifolia* prior to fructification at its natural density in the study area and plants kept on producing inflorescences and seeds, confirming that a minimum number of beetles per plant is necessary for the suppression of *A. artemisiifolia* population in the short term in the field (Guo et al. 2011, Zhou et al. 2014). Moreover, even if *O. communa* preferred *A. artemisiifolia* for feeding and oviposition

(Yamazaki et al. 2000, Cao et al. 2011), it was also found on non-target species, mainly belonging to Asteraceae family. The degree of damage was generally low as *O. communa* tends to move to its primary host to complete its life cycle. Observation of life stages other than adults on some resident plants confirms that, when *A. artemisiifolia* density is low, *O. communa* can potentially choose different plant species, suggesting that some attention should be paid to the risk for non-target species. Specific tests are currently being undertaken by the *Ophraella* task force (EU-project SMARTER) that is intensely evaluating the suitability of the beetle as a biological control in Europe.

Considering the two methods, it can be asserted that competitive vegetation using native flora plants has a small/null impact on ecosystems and it can be almost totally controlled by users. On the other hand, biological agents are often alien to the resident community and they potentially represent a risk for local flora, fauna and agricultural production. As for *O. communa*, preliminary results of a hazard analysis in France revealed a low risk for agriculture and the environment (Chauvel et al. 2017). Moreover, the beetle has been identified as one of the most promising agents for biocontrol of *A. artemisiifolia* in Italy, as a great overlap of *A. artemisiifolia* and *O. communa* suitable areas in current and future climatic scenarios was predicted (Lommen et al. 2017b, Sun et al. 2017). In addition, it is important to underline that the effect of the bio-agent can be more time and cost-saving than vegetation recovery, potentially ensuring an effective action on a wider area in a shorter time. Due to the possible risk for non-target species, an integrated control applying both of the two techniques should be monitored in the medium-long term as the insect use could conflict with the seeding of native mixture due to its possible attack on other Asteraceae beside *A. artemisiifolia*. In addition, in agricultural areas, the application of both competitive vegetation and biological control using *O. communa* are critical due to potential interferences on farming practices and *vice versa*, beyond issues related to optimising crop yields. However, agricultural areas probably represent the main sources of pollen and propagules of *A. artemisiifolia* as the species finds suitable conditions to persist due to repeated disturbance. Consequently, according to the current need for an even more sustainable agriculture, low impact solutions respecting alimentary products and environment should be developed.

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Supplementary material 1

Tables S1–S2, Figures S1–S2

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Explanation note: Table S1. Soil characteristics in the three investigated sites; Table S2. *O. communa* density on non-target species throughout summer 2015; Figure S1. *A. artemisiifolia* soil seed bank in the three sites; Figure S2. *A. artemisiifolia* traits in experimental plots.

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